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The inhibitory effect of Manuka honey on human colon cancer HCT-116 and LoVo cell growth. Part 2: Induction of oxidative stress, alteration of mitochondrial respiration and glycolysis, and suppression of metastatic ability

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Despite its high content of phenolic compounds, the chemopreventive activity of Manuka honey (MH) is still elusive. The aim of the present work was to evaluate the effects of MH on oxidative stress, antioxidant enzymes, cellular metabolism and the metastatic ability in HCT-116 and LoVo cells, paying particular attention to the molecular mechanisms involved. We observed a strong induction of oxidative stress after MH treatment since it augmented the accumulation of reactive oxygen species and increased the damage to proteins, lipids and DNA. Furthermore, MH suppressed the Nrf2-dependent antioxidant enzyme expression (superoxide dismutase (SOD), catalase and heme oxygenase-1) and the activity of SOD, catalase, glutathione peroxidase and glutathione reductase. Cell metabolisms were markedly disrupted after MH treatment. It decreased maximal oxygen consumption and spare respiratory capacity, which could reduce the mitochondrial function that is correlated with cell survival potential. Simultaneously, MH decreased the extracellular acidification rate (glycolysis) of HCT-116 and LoVo cells. Furthermore, MH suppressed the p-AMPK/AMPK, PGC1α and SIRT1 activation, involved in the survival of HCT-116 and LoVo cells under metabolic stress conditions. Dose-dependently, MH reduced the migration and invasion (MMP-2 and MMP-9) ability, and concurrently regulated EMT-related markers (E cadherin, N cadherin, and β-catenin) in both cell types. The above findings indicate that MH induces HCT-116 and LoVo cell death partly by enhancing oxidative stress, as well as by regulating the energy metabolism in both aerobic and anaerobic pathways and suppressing the metastatic ability.

1. Introduction

Colon cancer is the second most prevalent cause of death from cancer in men and women after lung cancer in the United States; in 2017, an estimated reported number of new cases of colon cancer was 71 420 in men and 64 010 in women and the number of deaths was 27 150 in men and 23 110 in women.[1] In Europe, it is the second most common cancer, with almost 500 000 new cases diagnosed accounting for 13.0% of all cancers and 214 866 deaths, which correspond to 12.2% of the total number of cancer deaths in 2012.[2]

Oxidative stress and disorder in the cellular redox equilibrium can enhance the possibility of various chronic diseases, including cancer, since they lead to damage of the main components of cells (lipids, proteins and DNA).[3] However, recent research has demonstrated that natural compounds have the properties to prompt ROS generation and suppress antioxidant enzyme activities, which promote oxidative stress inducing cancer cell death[4–10] and represent one of the important therapeutic regimes in conventional therapeutic management.

In recent cancer research, the significance of mitochondria as oxygen sensors and producers of ATP has become central, and researchers have demonstrated that mitochondrial metabolism plays an important role linked to faster proliferation of

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several types of cancer cells.[11] Cancer cells modulate various signaling pathways to overcome metabolic stress. AMP-activated protein kinase (AMPK), a recognized sensor for metabolic stress, is activated when cellular ATP levels decrease. This activation enhances the catabolic pathway activity for generating higher ATP levels and inhibits the anabolic pathways from inducing a favorable environment for cancer cell growth.[12,13]

Epithelial–mesenchymal transition (EMT) and matrix metalloproteinases (MMPs) are the essential events for cancer cells since migration and invasion are required for the progression of metastatic cancer.[14–16]

The most important concern about anti-cancer drugs is their probable toxicity after treatment. At present, natural compounds are streamlined because of their potential toxicity to cancer cells and because they are less or non-toxic to non-cancer cells.[7,17]

The bioactive components of Manuka honey (MH) are thought to be accountable for its anti-microbial and wound-healing capacities[18,19], but its chemopreventive properties remain to be determined in spite of its high phenolic compounds.[20,21] In order to investigate whether the use of MH could be useful for the treatment and/or prevention of colon cancer, we evaluated the effect of MH on ROS, oxidative stress biomarkers (lipid, protein and DNA), antioxidant enzyme activity and expression, as well as oxidative phosphorylation (OXPHOS) and glycolysis in human colon adenocarcinoma cells (HCT-116) and Dukes' type C, grade IV, colon metastasis (LoVo) cells. Considering the role of the AMPK pathway in cell metabolism, we also investigated the effect of MH on the expression of AMPK associated downstream proteins in both cell types. In addition, we aimed to confirm the role of MH in colorectal metastasis and related molecular mechanisms *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

MH samples (from New Zealand) were imported by EfitSrl (Italy) and kept at 4 °C until analysis. All chemicals and reagents were purchased from Sigma-Aldrich (Milan, Italy). Media for cell culture were obtained from Carlo Erba Reagents (Milan, Italy).

2.2. Cell culture

Human colon adenocarcinoma (HCT-116) and Dukes' type C, grade IV, colon metastasis (LoVo) cell lines were bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCT-116 cells and LoVo cells were cultured in McCoy's 5A and F-12 K media, respectively. All the media were prepared with 100 IU mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 10% heat-inactivated fetal bovine serum. All cell lines were preserved in an incubator at 37 °C in a humidified atmosphere (95% air, 5% CO_2) and the cells were used between the 6^{th} and 10^{th} passages for the subsequent experiments.

2.3. Determination of intracellular ROS levels

Intracellular ROS accumulation was determined with the CellROX® Oxidative Stress kit (Invitrogen™, Life Techonoliges, Milan, Italy) as previously described by Giampieri *et al.*[22] The cells were seeded at a density of 1.5×10^5 cells per well in 6 wells and treated for 48 h with MH (0, 10, 15 and 15 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells). Then, the cells were trypsinized and centrifuged for 10 min at 1500 rpm. Next, the CellROX® Orange reagent (2 μL mL^{-1}) was added and the cells were incubated at 37 °C for 30 min. Again, the cells were centrifuged for 10 min at 1500 rpm to remove the excess dye and medium, and then resuspended in 100 μL of PBS. The cells were analyzed by using a Tali® Image-Based cytometer (Invitrogen™, Life Techonoliges, Milan, Italy) and the results were expressed as the fold change. The cells are fluorescent in an oxidised state and non-fluorescent in a reduced state.

2.4. Determination of antioxidant enzyme activity and biomarkers of oxidative stress

Both colon cancer cells were treated with several concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The cellular lysates were prepared by using the RIPA buffer (Sigma-Aldrich, Milan, Italy) for antioxidant enzyme activity and oxidative marker (lipids and proteins) determination. The antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) were assessed by spectrophotometric methods as previously reported by Giampieri *et al.*[23] The thiobarbituric acid-reactive substances (TBARS) and protein carbonyl content were quantified as the biomarkers of lipid and protein oxidative damage according to the method previously described.[23]

2.5. Bioenergetic assay

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed with an Agilent Seahorse XF24 Analyzer (Seahorse Bioscience, North Billerica, MA, USA) according to Giampieri *et al.*[22] and Shen *et al.*[24] representing the OXPHOS and glycolysis of the HCT-116 and LoVo cells in diverse circumstances, respectively. The HCT-116 and LoVo cells were seeded in an XF24 cell culture plate at a density of 3.0×10^4 cells per well and allowed to adhere for 20 to 24 h. After that, both cells were treated with several concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. At the end of the incubation time, the medium was replaced with 500 μL per well of XF assay media (supplemented with 25 mM glucose, 2 mM glutamine, and 1 mM sodium pyruvate, without serum). The Agilent Seahorse XF24 Analyzer allows for the sensitive measurement of multiple parameters of mitochondrial respiration, including the basal OCR, spare respiratory capacity (SRC), maximum respiration rate (MRC), ATP-linked respir-

ation and proton leak, and glycolytic function from intact cultured cells.

After the baseline measurements, the OCR was measured after the sequential addition of 55 μL of oligomycin (an inhibitor of complex V), 61 μL of 2,4-dinitrophenol (2,4-DNP) (allowing the protons to leak across the inner mitochondrial membrane and thus bypass ATP synthase) and 68 μL of rotenone/antimycin (inhibitors of complex I and complex III), to reach the working concentrations of 3 μg ml^{-1}, 300 μM and 1 μM/10 μM, respectively. The following equation was used to determine the specific parameters: ATP-linked respiration = Basal OCR value − Oligomycin OCR value, Proton leak = Oligomycin OCR value − Rotanone/Antimycin OCR value, SRC = 2,4-DNP OCR value − Basal OCR value and MRC = 2,4-DNP OCR value − Rotanone/Antimycin OCR value. The OCR was reported in picomoles per minute (pmole per min) per 3.0×10^4 cells and all data were reported as a mean value of three independent analyses ± standard deviation (SD).

The ECAR was measured after the addition of 55 μL of rotenone (1 μM), 61 μL of glucose (30 mM) and 68 μL of 2-deoxy-D-glucose (2-DG) (100 mM). The following equation was used to determine the specific parameters: Glycolysis = Glucose ECAR value − Basal ECAR value, Glycolytic capacity = Glucose ECAR value − 2-DG ECAR value and Glycolytic reserve = the amount of glycolytic capacity − the amount of glycolysis. The ECAR is reported in milli-pH per minute (mpH per min) per 3.0×10^4

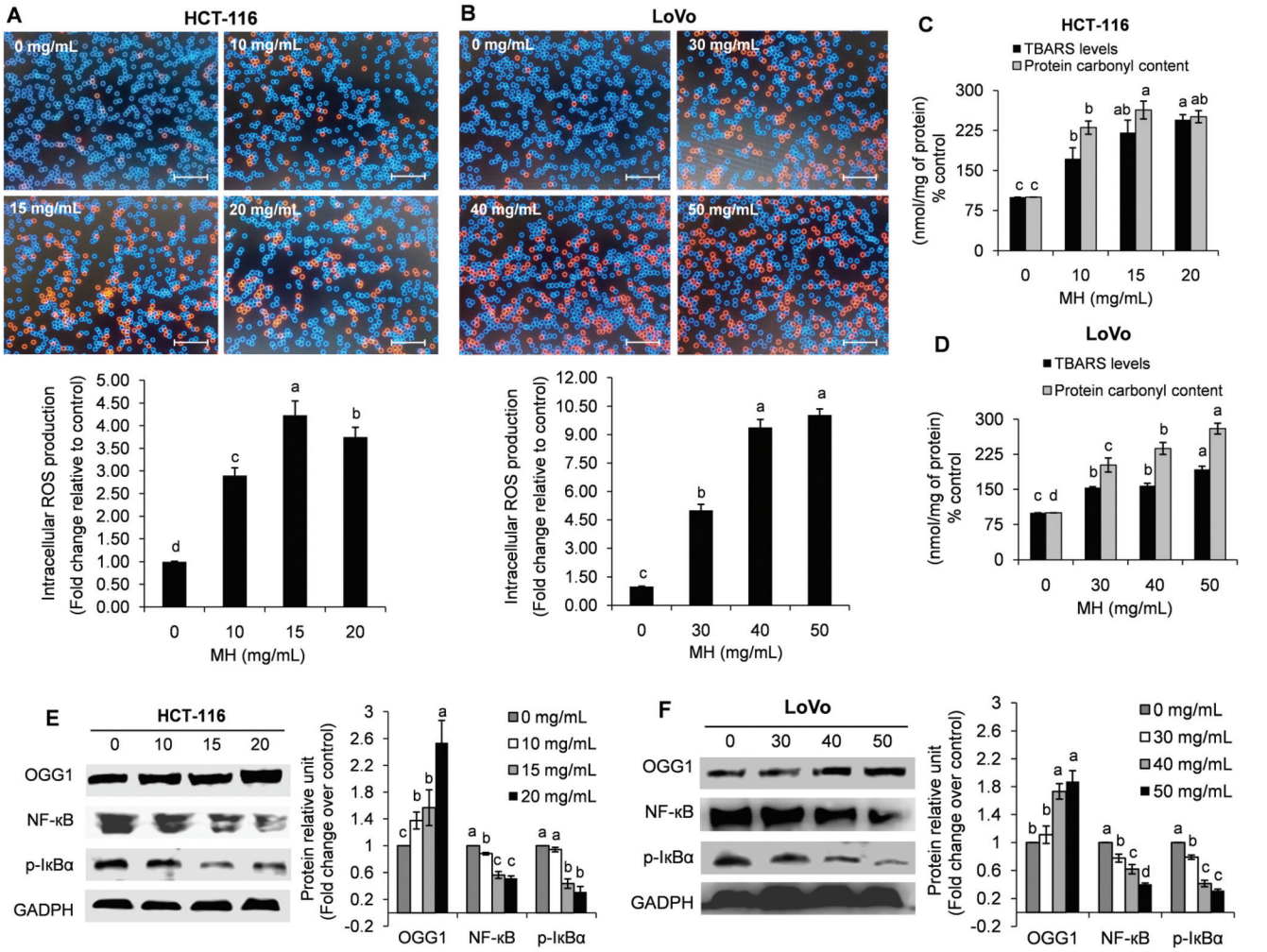

Fig. 1 MH induces ROS generation and oxidative stress in HCT-116 and LoVo cells. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. Intracellular ROS levels were determined by using the CellROX® Orange assay kit and the Tali™ Image-based Cytometer in (A) HCT-116 and (B) LoVo cells. Representative fluorescence images of HCT-116 cells show the effect of MH treatment: the blue color corresponds to live cells and red color corresponds to the ROS-induced cells. Scale bar: 50 μm. The lipid and protein oxidative damage was evaluated by assessing the TBARS levels and protein carbonyl content in (C) HCT-116 and (D) LoVo cells. The results were measured as mmol per mg of protein and expressed as the percent of the control. The protein expressions of OGG1, NF-κB and p-IκBα were analyzed by western blotting in (E) HCT-116 and (F) LoVo cells. GADPH was utilized as a loading control. All data are indicated as the mean ± standard deviation (SD) ($n = 3$). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

cells and all data were reported as a mean value of three independent analyses ± SD.

2.6. Colony formation assay

The colony formation assay was performed according to the method described by Waghela *et al.* with slight modification.[25] Briefly the cells were seeded at a density of 5×10^5 cells per well in 6 wells and allowed to adhere for 20 to 24 h. After that, the cells were treated with MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. After the incubation time, the cells were trypsinized and 1000 cells were seeded in a 6-well plate. The cells were allowed to grow for 10 to 12 days until small colonies were visible. The colonies were fixed with methanol and stained with 0.2% methylene blue stain. The plating efficiency (PE) was considered by the ability of a single cell to survive and to grow in the form of a colony. The PE was defined by the following formula: Percentage PE = (Number of colonies formed/Number of cells seeded) × 100. All data were reported as a mean value of three independent analyses ± SD.

2.7. Wound assay

The wound assay was performed by a slightly modified method as previously described by Amatori *et al.*[26] A linear wound was produced in HCT-116 and LoVo confluent cells by scratching the bottom of the plate with a 200 μL sterile pipette tip. After the scratch, the cells were washed two times with PBS and incubated with specific media for each cell containing 2% serum. Microphotographs of the wounds were taken with a light microscope LeitzFluovert FU (Leica Microsystems) at zero time and after 48 h incubation with the different MH treatments (0, 10, 15 and 15 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells). After the incubation time, the cells were fixed with methanol and stained with 0.2% methylene blue stain. After the photographs were taken, the wound areas were analyzed using NIH Image J software in order to calculate the percentage of wound closure after the treatments.

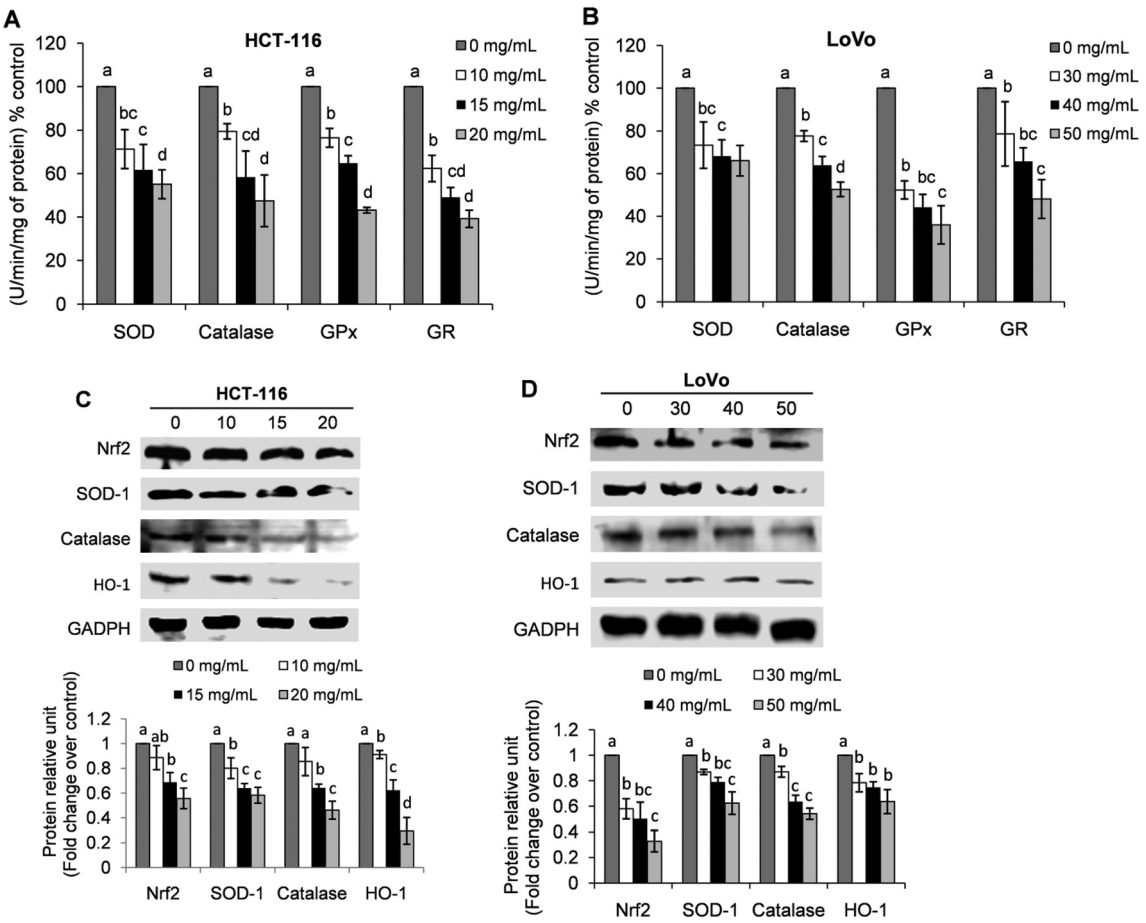

Fig. 2 MH suppresses the antioxidant enzyme activity and expression in HCT-116 and LoVo cells. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. SOD, Catalase, GR and GPx activities were expressed as the unit per mg protein and the results were expressed as the percent of the control in (A) HCT-116 and (B) LoVo cells. Nrf2, SOD-1, CAT and HO-1 protein expressions were analyzed by western blotting in (C) HCT-116 and (D) LoVo cells. GADPH was utilized as a loading control. All data are indicated as the mean ± SD ($n = 3$). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

2.8. Immunoblotting analysis

After the specific treatment, the HCT-116 and LoVo cell pellets were collected and protein lysates were prepared by using lysis buffer (120 mmol L^{-1} NaCl, 40 mmol L^{-1} Tris [pH 8], and 0.1% NP40) containing protease inhibitor cocktails (Sigma) and centrifuged at 13 000*g* for 15 min. The proteins from the cell supernatants were separated on 8 or 10% polyacrylamide gel, and transferred into a nitrocellulose membrane, with the trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). Tris HCI buffered saline containing Tween 20 (TBST) with 5% non-fat-milk were used for blocking the membrane at room temperature for 1 h. 8-Oxoguanine DNA glycosylase (OGG1), nuclear factor kappa B (NF-κB), phosphorylated inhibitor of kappa B (p-IκBα), nuclear-related factor 2 (Nrf2), SOD, Catalase, heme oxygenase 1 (HO-1), AMPK, phosphorylated (p)-AMPK, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), sirtuin 1 (SIRT1), MMP-2, MMP-9, E-cadherin, N-cadherin, β-catenin and glyceraldehyde-3-phosphate dehydrogenase (GADPH) primary antibodies (1 : 500 dilutions) were used after overnight incubation at 4 °C. After incubation, the membranes were washed with TBST (3 times) and again incubated with their specific alkaline phosphatase conjugated secondary antibodies (1 : 80 000) for another 1 h. The immunolabeled proteins were identified by using a chemiluminescence method (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany) and the bands were quantified using Image Studio Digits Software 3.1 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

2.9. Statistical analysis

The statistical analysis was assessed by using the STATISTICA software (Statsoft Inc., Tulsa, OK, USA) and the results are expressed as the mean values with a SD of three independent experiments. The significant differences are characterized by letters obtained from one-way analysis of the variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test ($p < 0.05$).

3. Results and discussion

3.1. MH induces intracellular ROS production and oxidative stress in HCT-116 and LoVo cells

In our previous work, we evaluated the anti-proliferative effects of MH on HCT-116 and LoVo cells.[20] Here we aimed to explore

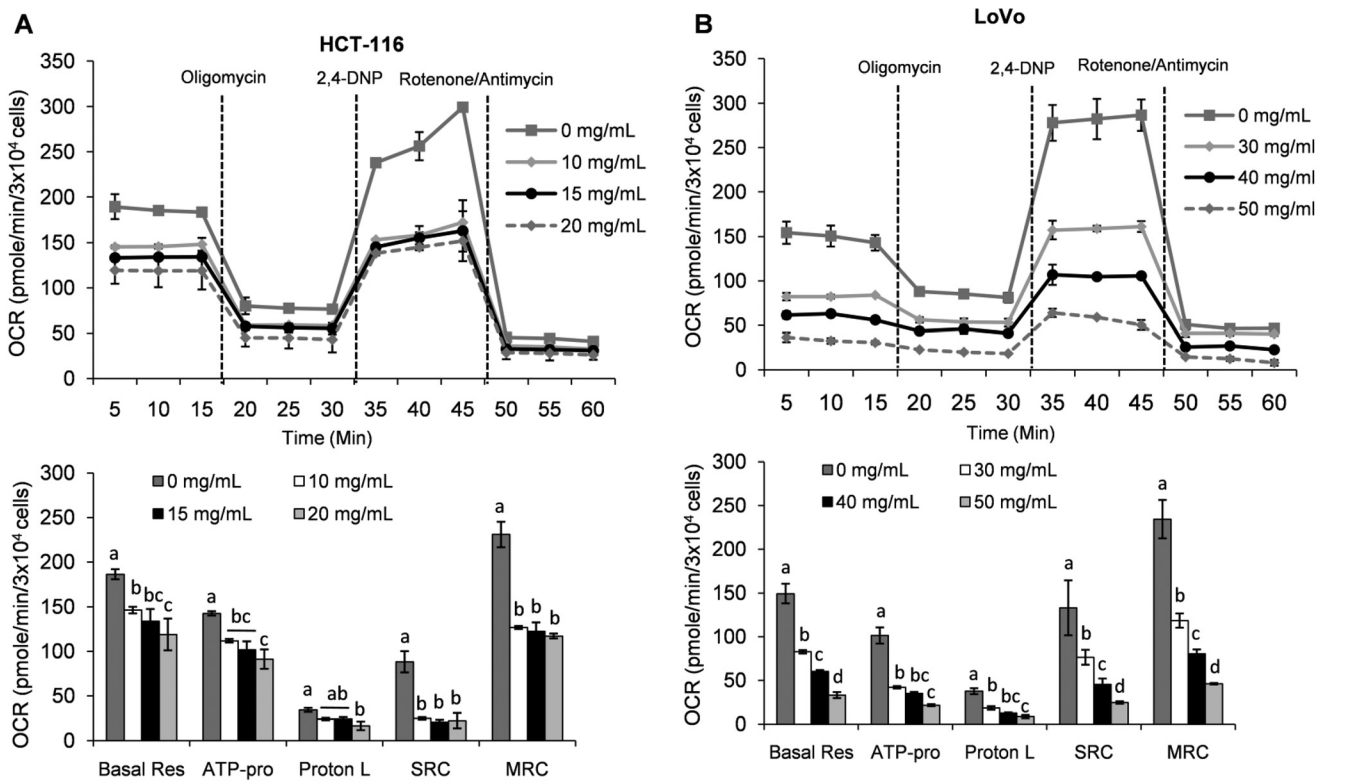

Fig. 3 Regulation of mitochondrial respiration by MH in HCT-116 and LoVo cells. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The OCR was determined by using the Seahorse XF-24 Extracellular Flux Analyzer after the following sequential injections: oligomycin (3 μg ml^{-1}), 2,4-DNP (300 μM), and rotenone/antimycin (1 μM/10 μM) in (A) HCT-116 and (B) LoVo cells. The basal respiration, ATP production, proton leak, maximal respiration capacity and spare respiration capacity were calculated from the XF cell Mito stress test profile in both cell lines. All data are indicated as the mean ± SD (n = 3). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$). Basal Res, basal respiration; ATP-pro, ATP production; Proton L, proton leak; SRC, spare respiration capacity; MRC, maximal respiration capacity.

whether MH induces ROS production and oxidative stress for inducing colon cancer cell death. We found that MH treatments significantly ($p < 0.05$) increased intercellular ROS accumulation in HCT-116 and LoVo cells in a dose-dependent manner (Fig. 1A and B). ROS exhibit dual effects *in vitro*, depending on their concentration, and they act as a redox messenger under normal physiological conditions, while in cancer cells they induce apoptosis for promoting cell death.[27] A large number of studies have indicated that ROS are moderators of intracellular signaling cascades and can activate a sequence of pathways for programmed cancer cell death after exposure to various natural compounds.[5,7,28]

In colon cancer HCT-15 and HT-29 cells, Indian commercial honey increased ROS generation to induce apoptotic cell death at 30 mg mL^{-1}.[4] Additionally, strawberry tree honey induced a high amount of ROS in HCT-116 and LoVo cells at 9 mg mL^{-1} and 20 mg mL^{-1}, respectively.[20] In our results, we evaluated that MH at 15 mg mL^{-1} for HCT-116 cells and 50 mg mL^{-1} for LoVo cells (Fig. 1A and B) generated a high amount of ROS. Furthermore, in HCT-116 and DLD-1 colon cancer cells, chrysin and hydroxytyrosol treatment increased cytosolic and mitochondrial ROS generation to activate apoptosis.[7,9] Similarly, quercetin (highly present in MH) increased the ROS associated intrinsic apoptotic and ER stress pathway in colon cancer HT-29, HCT-116 and HCT-15 cells.[29,30]

Next, we measured the biomarkers of oxidative damage to lipids (TBARS), protein carbonyl content and DNA (OGG1 expression) after MH treatment (Fig. 1). In oxidative stress, ROS are continuously produced and cause lipid, protein and DNA damage which leads to cancer cell death by inducing apoptosis or the necrotic mechanism.[27] Our results showed that the TBARS levels and protein carbonyl content, which are oxidative products, significantly ($p < 0.05$) increased from 137 to 245% and 130 to 263% in HCT-116 cells and from 138 to 192% and 127 to 279% in LoVo cells after MH treatment compared to the control (Fig. 1C and D). In this context, several authors reported that natural polyphenols exert oxidative stress inducing cancer cell death by increasing TBARS and protein carbonyl levels.[5,7,29] For example, quercetin derivatives, *p*-methoxycinnamic acid and 5-hydroxy-7-methoxyflavone increased the oxidative marker TBARS and protein carbonyl levels in HT-29 and HCT-116 colon cells to induce apoptotic cell death.[5,7,29]

OGG1, a bifunctional glycosylase, has the ability of splitting the glycosidic bond in the mutagenic lesion and consequently, breaking the strand of the DNA backbone.[31] Recent reports have indicated that, in advanced stages of colorectal cancer (CRC) patients, the OGG1 gene expression was downregulated.[32] Western blot analysis revealed that MH treatment increased the protein expression of OGG1 from 1.3 to 2.5 fold in HCT-116 cells (Fig. 1E) and from 1.1 to 1.8 fold in

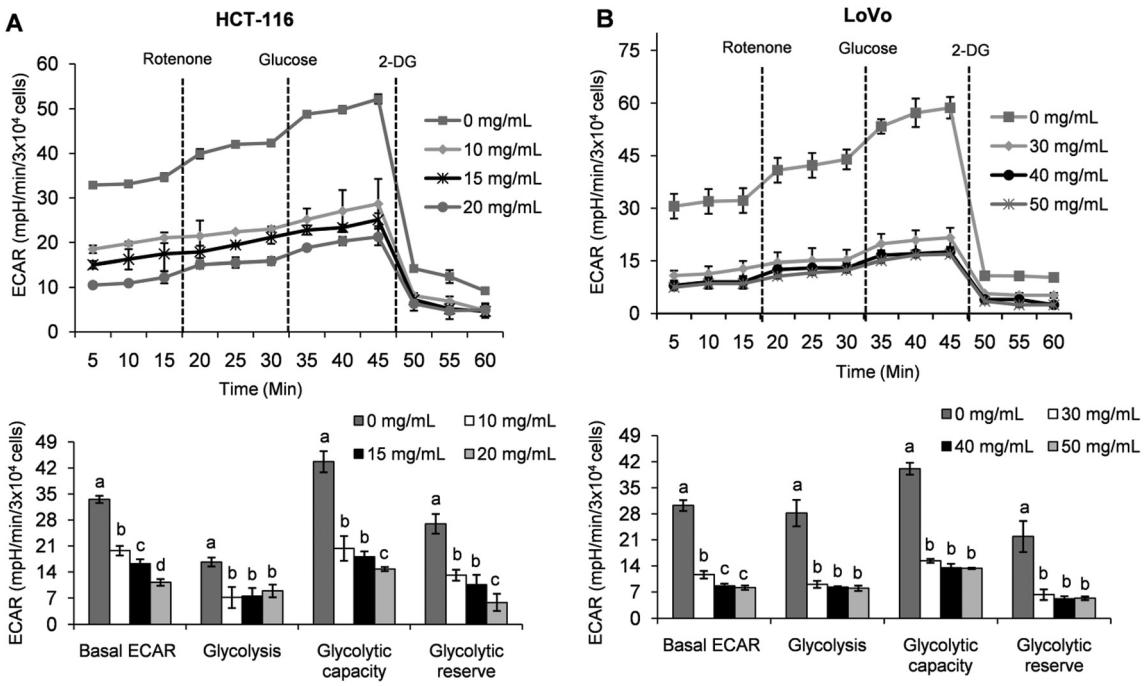

Fig. 4 Regulation of glycolysis by MH in HCT-116 and LoVo cells. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The ECAR was determined by using the Seahorse XF-24 Extracellular Flux Analyzer after the following sequential injections: rotenone (1 μM), glucose (30 mM), and 2-DG (100 mM) in (A) HCT-116 and (B) LoVo cells. The basal ECAR, glycolysis, glycolytic capacity and glycolytic reserves were calculated from the XF glycolysis stress test profile. All data are indicated as the mean ± SD ($n = 3$). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

LoVo cells (Fig. 1F) compared to the control, indicating DNA damage.

Interestingly, in a previous study from our group, we observed that MH has the ability to protect healthy non-cancer cells from oxidative stress by decreasing ROS generation and increasing lipid and protein damage.[19] These findings suggest that the anti-proliferative effect of MH is associated with the oxidative stress-induced damage of human colon cancer HCT-116 and LoVo cells.

3.2. Effect of MH on NF-κB signaling in HCT-116 and LoVo cells

The protein expression of NF-κB and p-IκBα in both colon cancer cells are shown in Fig. 1E and F. In oxidative stress, NF-κB can be activated by phosphorylation of IκBα which initiates an inflammatory response.[33] Our results showed that MH treatment suppressed the expression of NF-κB and p-IκBα in HCT-116 and LoVo cells in a dose-dependent way (Fig. 1E and F). Aberrant NF-κB activation has been detected in colorectal and colitis-associated tumors, increasing cell proliferation, anti-apoptosis, genomic instability, glycolysis and drug resistance in colon cancer cells.[34] Our results are very similar to those obtained in HT-29 cells in which a greater amount of ROS generation and suppressed NF-κB and p-IκBα expression was also found after plant polyphenol treatment.[35]

3.3. MH regulates antioxidant levels in HCT-116 and LoVo cells

The response of antioxidant enzyme activity such as SOD, catalase, GR and GPx is reported in Fig. 2A and B. The antioxidant system maintains the cellular redox reaction by scavenging ROS and reducing the oxidized glutathione (GSH/GSSG) or NADPH/$NADP^+$ ratio, but in oxidative stress, the antioxidant defense system is damaged due to excess ROS.[3] In our work, the activity of antioxidant enzymes was depleted compared to the control after MH treatment in both CRC cell lines (Fig. 2A and B), leading to the accumulation of more ROS associated cell damage. Raja *et al.*[30] and Gunasekaran *et al.*[5] also reported that decreased SOD, catalase and GPx were observed in HT-29, HCT-15 and HCT-116 colon cancer cells after flavonoid treatment used to activate mitochondrial-dependent apoptosis. Additionally, quercetin derivatives decreased the antioxidant enzymes SOD, catalase, and GPx and the GR activity in HT-29 and HCT-15 colon cancer cells to induce cytotoxic effects.[10]

Furthermore, protein expressions of the transcription factor Nrf2 and its synchronized antioxidant enzymes SOD, catalase

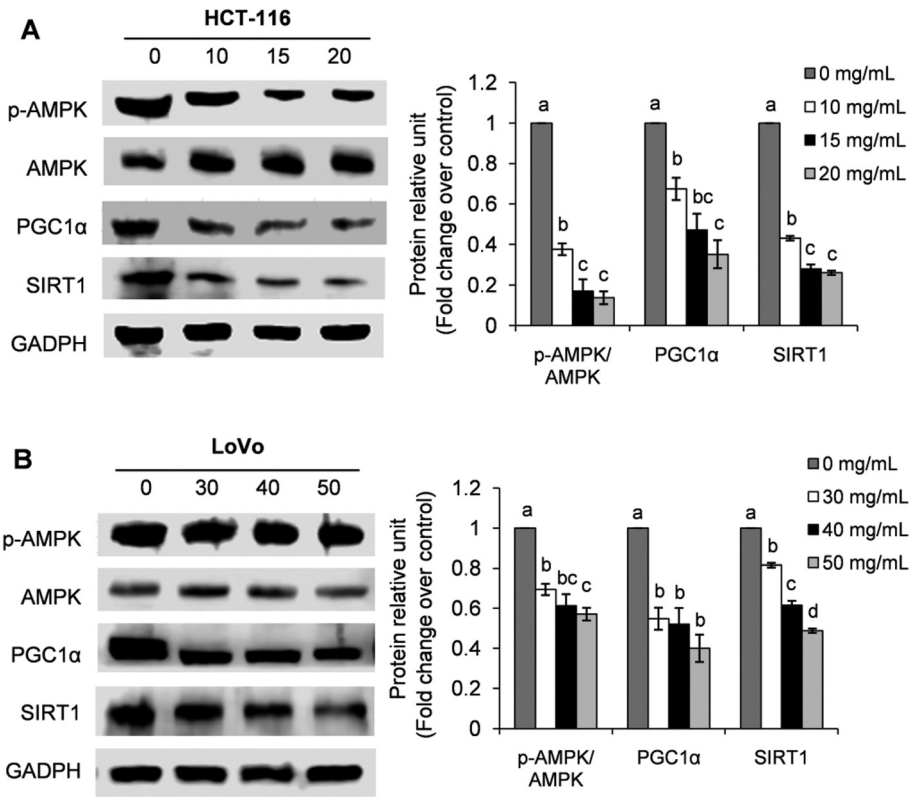

Fig. 5 MH suppresses the cellular biogenesis by targeting the AMPK pathway. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The protein expressions of p-AMPK/AMPK, PGC1α and SIRT1 were determined by western blot analysis in (C) HCT-116 and (D) LoVo cells. GADPH was utilized as a loading control. All data are indicated as the mean ± SD (n = 3). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

and HO-1, were found to be lowered after MH treatment at different concentrations compared to the control in both tested colon cancer cells (Fig. 2C and D). Nrf2 has been considered as a tumor suppressor for its cytoprotective effects against oxidative stress as it controls the expressions of SOD, catalase, HO-1 and glutathione.[36] Conversely, several recent studies revealed that the hyperactivation of Nrf2 signaling creates a favorable environment for the survival of malignant cells, protecting them against oxidative stress, chemotherapeutic agents and radiotherapy.[37] Earlier reports have suggested that biologically active compounds from plants (quercetin, 5-hydroxy-7-methoxyflavone and trigonelline) induce anticancer properties by suppressing the expression of the Nrf2 gene and other antioxidant enzymes such as thioredoxin, peroxiredoxin, GR, catalase and SOD in colon (HCT-116 and DLD-1) and liver (Hep3B) cancer cells due to oxidative stress.[6,7,28,29] Moreover, in non-malignant cells, MH treatment increases the antioxidant enzyme activity (SOD and catalase) and expressions (Nrf2, SOD and catalase) to combat oxidative stress-induced damage.[19] These findings highlight that MH treatment decreases the antioxidant enzyme activity and expression which further elevates ROS generation.

3.4. Effect of MH on oxidative phosphorylation and glycolysis in HCT-116 and LoVo cells

Metabolic changes are one of the hallmarks in the development of tumors. Depending on the energetic metabolism, the chemopreventive and therapeutic strategies are different in different tumors. Cancer cells have the ability to reprogramme the metabolic profile by the metabolic shift from mitochondrial OXPHOS to aerobic glycolysis in order to maintain the requested energy level for continuous growth, rapid proliferation, and other typical characteristics.[38] In this perspective, we used a novel extracellular flux analyzer to evaluate the several parameters of mitochondrial respiration or the OCR and glycolysis or ECAR in order to investigate the bioenergetic characterization of human colon cancer HCT-116 and LoVo cells after MH treatment.

MH treatment at different concentrations decreased the basal levels of mitochondrial respiration, the proton leak, SRC and MRC, and this was mainly due to the decreased ATP-linked respiration in the mitochondria (Fig. 3). The results showed that MH treatment significantly ($p < 0.05$) reduced the basal OCR in a dose-dependent manner up to 119.00 ± 10.82 pmol per min per 3×10^4 cells for HCT-116 cells and 33.17 ± 3.45 pmol per min per 3×10^4 cells for LoVo cells compared to the control cells (Fig. 3A and B). Furthermore, MH treatment also suppressed the activity of ATP-linked respiration, the proton leak, the SRC and MRC up to 91.29 ± 10.08, 16.63 ± 4.38, 22.39 ± 0.25 and 117.27 ± 0.50 pmol per min per 3×10^4 cells, respectively, compared to the control in HCT-116 cells (Fig. 3A). Similarly, in LoVo cells the activity was suppressed up to 21.56 ± 1.18, 8.60 ± 1.84, 24.62 ± 0.138 and 46.18 ± 0.81 pmol per min per 3×10^4 cells, respectively, compared to the control (Fig. 3B).

Regarding the ECAR, we observed that MH treatment reduced the basal values by 23.52 ± 0.87 to 10.51 ± 0.90 mpH per min per 3×10^4 for HCT-116 cells and 13.38 ± 0.87 to 8.18 ± 0.58 mpH per min per 3×10^4 for LoVo cells compared to the control (Fig. 4). In addition, in both cell lines MH treatment markedly reduced glycolysis (up to 14.82 ± 0.55 and 8.01 ± 0.71 mpH per min per 3×10^4 cells), glycolytic capacity (up to 7.52 ± 1.10 and 13.36 ± 0.19 mpH per min per 3×10^4 cells) and gylcolytic reserve (up to 5.89 ± 2.20 and 5.35 ± 0.47 mpH per min per 3×10^4 cells) compared to the control in a dose-dependent manner (Fig. 4A and B).

Our results are in line with earlier studies that confirmed the ability of natural compounds to suppress the parameters of mitochondrial respiration[22,24] and glycolysis[39] in several

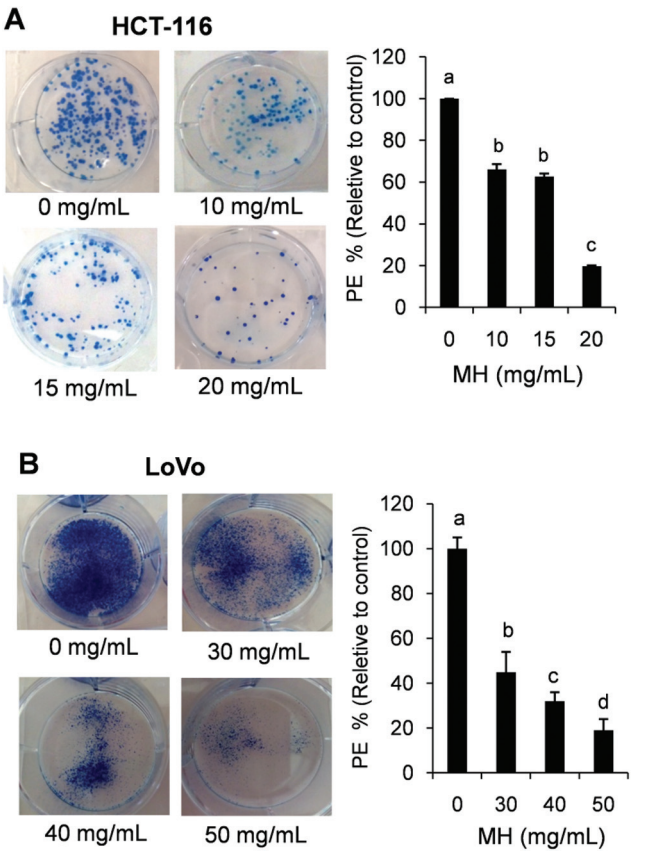

Fig. 6 Inhibition of the colony formation ability by MH in HCT-116 and LoVo cell lines. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The cells were seeded at low density in McCoy's 5A and F-12 K media supplements with different concentrations of MH for 12 days. The results were expressed as a % of the plating efficiency that corresponds to untreated cells. The colonies were subsequently fixed with 70% ethanol and stained with methylene blue for the analysis of colony formation. Quantitative image analysis of colonies in cultured (A) HCT-116 and (B) LoVo cells. All data are expressed as the mean ± SD ($n = 3$). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

cancer cells in order to induce chemopreventive effects. To date, no studies have been performed on the effect of MH in colon cancer cell metabolism targeting both oxidative phosphorylation (OXPHOS) and glycolysis pathways. Thus, all data suggest that MH is a regulator of energy metabolism both in the aerobic and anaerobic pathways in HCT-116 and LoVo cells.

3.5. Effect of MH on AMPK signaling pathway

Western blot analysis was performed to investigate the expression of proteins related to the AMPK pathway and its downstream targets PGC1α and SIRT1 in HCT-116 and LoVo cells after treatment with different concentrations of MH (Fig. 5A and B). The AMPK signaling pathway is known to be involved in promoting tumor cell survival by increasing mitochondrial biogenesis and inducing metabolic adaptation under glucose-limiting conditions.[12] However, recent evidence suggests that AMPK activation is necessary to regulate oxidative stress and enhance cancer cell proliferation in a metabolic stress microenvironment.[12,13] In the present study, we showed that MH treatment significantly decreased ($p < 0.05$) the p-AMPK expression in both cell types compared to the control (Fig. 5A and B). Kim *et al.* explained that quercetin (an important flavonoid present in MH) suppressed AMPK activation both in *in vitro* and *in vivo* on colon cancer models under hypoxic conditions.[40] Therefore, the suppression of AMPK might also be a therapeutic approach for cancer treatment. The activation of AMPK increases the expression of downstream targets PGC-1α and its associated genes so that cancer cells survive under energetic stress conditions.[12] Our data suggest that MH treatment suppressed the PGC-1α expression in HCT-116 and LoVo cells compared to the control in a dose-dependent manner (Fig. 5A and B). SIRT1 is another downstream target of AMPK, depending on the nature and phase of the tumor; it acts as a tumor suppressor and an oncoprotein.[41] In CRC patients, elevated levels of SIRT1 expression promote carcinogenesis and are associated with a poor prognosis.[42] In the present study, we found that MH treatment suppressed the expression of SIRT1 in both colon cancer cells at different concentrations (Fig. 5A and B). Overall, these results suggested that MH treatment decreased AMPK activation leading to a diminution of PGC1α and SIRT1 expressions, which hinders HCT-116 cell survival by inhibiting stress adaptation. In contrast, in non-malignant cells, MH treatment improved the AMPK expression together with PGC1α and SIRT1 in order to decrease the stress-induced damage.[19]

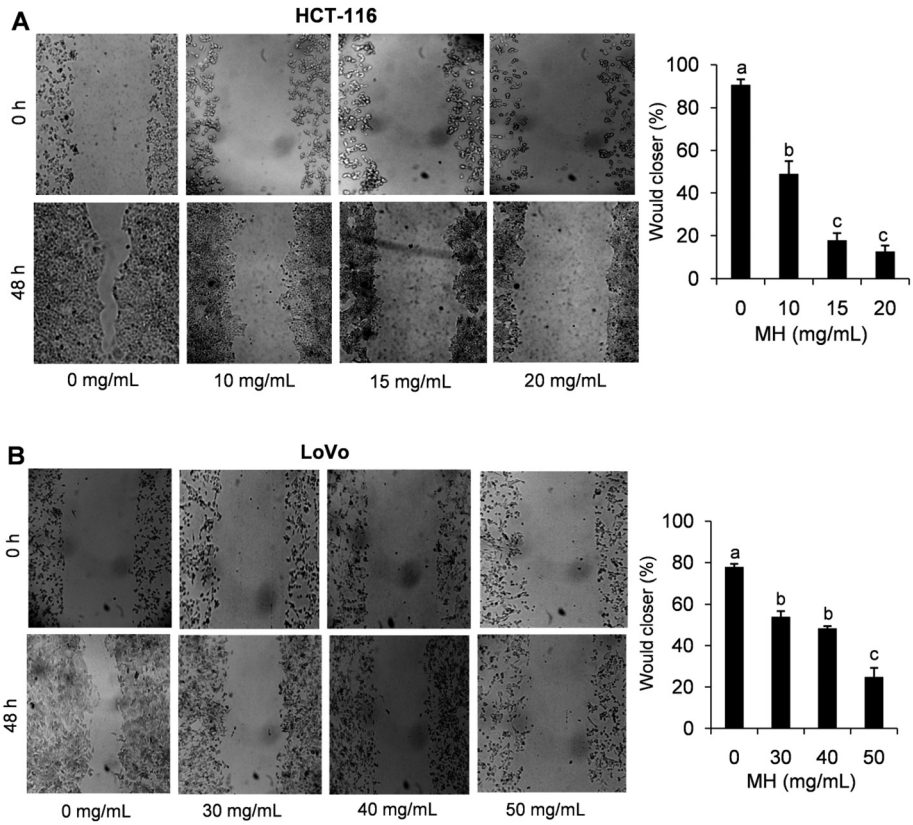

Fig. 7 MH reduces the migration ability of HCT-116 (A) and LoVo (B) cells by wound healing assay. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The wound closure percentages were analyzed by Image J software. All data are indicated as the mean ± SD ($n = 3$). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

3.6. MH diminishes colony formation ability of HCT-116 and LoVo cells

As shown in Fig. 6, different colony formation capabilities were observed in different colon cancer cells. Chronic exposure of MH decreased colony formation from 26% to 79% at a concentration of 10 to 20 mg mL^{-1} in HCT-116 cells (Fig. 6A) and 55% to 80% at a concentration of 30 to 50 mg mL^{-1} in LoVo cells (Fig. 6B) compared to untreated cells (100%). Several natural compounds were used to observe the colony formation assay on cancer cells. In this context, the chronic exposure of pterostilbene reduced colony formation up to 40% and 85% in colon cancer HCT-116 and HT-29 cells,[43] while millet bran derivatives decreased colony formation up to 75% in DLD-1 and HCT-116 colon cancer cells, respectively.[28]

3.7. Effect of MH on migration and invasion ability of HCT-116 and LoVo cells

In addition, we evaluated the inhibitory effects of MH on the migration and invasion in colon cancer HCT-116 and LoVo cells. As shown in Fig. 7, after 48 h of culture, both untreated cells were able to migrate and partially fill the empty wound area. MH treatment reduced the migration ability of HCT-116 cells from 37% to 88% compared to the control (10%) and in LoVo cells, from 30% to 79% compared to the control (15%) in a dose-dependent manner (Fig. 7A and B). In this context, Kee *et al.* evaluated that quercetin suppressed colon cancer CT-26 cell migration in a dose-dependent way.[44] Similarly, several bioactive compounds have the ability to suppress the migration in different cancer cells *in vitro* so as to induce anti-metastasis effects.[26,45,46] In contrast, in a recent study, it was found that MH promotes a wound healing mechanism in human dermal fibroblasts due to the improvement of the antioxidant response by modulating the AMPK/Nrf2 signaling pathway,[19] suggesting that MH effects depend on the cell type.

Several studies have found that the expression and activation of MMP-2 and MMP-9 in CRC is associated with cancer progression, angiogenesis, invasion and metastasis.[16] Therefore, we observed the MMP-2 and MMP-9 protein expression by western blot (Fig. 8). In HCT-116 cells, MH suppressed the expression of both invasion proteins up to 0.50 fold at concentration of 15 to 20 mg mL^{-1}, while in LoVo cells it is suppressed up to 0.28 fold at a concentration of 50 mg mL^{-1} (Fig. 8). Moskwa *et al.* showed that the activity and the expression of MMP-2 and MMP-9 decreased in glioblastoma cells when using Polish honey.[47] Some recent investigations also observed the anti-metastatic effects of natural compounds through suppressing the invasion ability by down-regulating the MMP-2 and MMP-9 activity or expression in several *in vitro* cancer models.[44–46]

Migration and invasion are essential steps in the progression of cancer metastasis and we evaluated that MH treat-

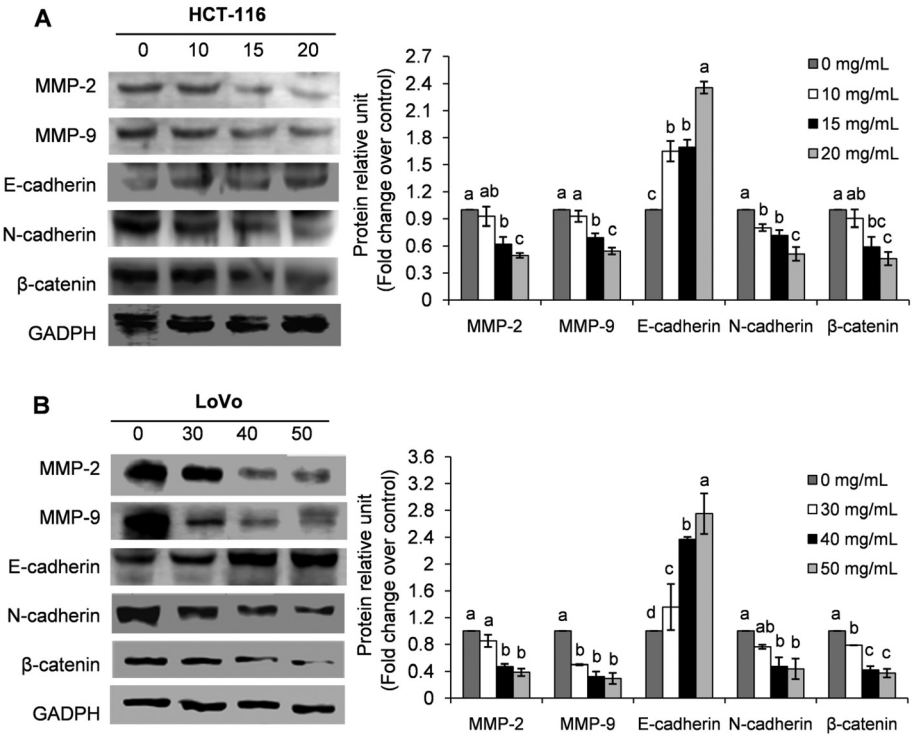

Fig. 8 MH regulates the expression of the invasion and EMT-related genes in HCT-116 and LoVo cells. Both cells were treated with different concentrations of MH (0, 10, 15 and 20 mg mL^{-1} for HCT-116 cells and 0, 30, 40 and 50 mg mL^{-1} for LoVo cells) for 48 h. The protein expressions of MMP-2, MMP-9, E-cadherin, N-cadherin and β-catenin were determined by western blotting analysis in (A) HCT-116 and (B) LoVo cells. GADPH was used as a loading control. All data are indicated as the mean ± SD (n = 3). The columns associated with the same set of data with different symbolic letters are significantly different ($p < 0.05$).

ment significantly suppressed both parameters in HCT-116 and LoVo colon cancer cells (Fig. 8).

3.8. Effect of MH on the expressions of EMT-related genes in HCT-116 and LoVo cells

EMT markers, such as E-cadherin, N-cadherin and β-catenin contribute a significant role in the progression of cancer metastasis, increasing the migration and invasion by connecting more stromal-cellular or cell-matrix adhesion.[15] In this step, we examined the effect of MH on the expression of EMT-related markers of E-cadherin, N-cadherin and β-catenin in colon cancer HCT-116 and LoVo cell lines (Fig. 8A and B). As shown in Fig. 8, with MH the expression of E-cadherin increased up to 2.36 fold and 2.75 fold for both cells, whereas the expression of N-cadherin and β-catenin decreased up to 0.50 fold and 0.44 fold for HCT-116 cells and 0.46 fold and 0.37 fold for LoVo cells, respectively, compared to the control. Kee *et al.* reported the anti-metastatic effects of quercetin in CRC CT-26 cells by modulating the expression of the EMT-related genes (N-cadherin, β-catenin and Snail).[44] There is an growing amount of evidence on the anti-metastatic effects of phenolic compounds, with particular attention being given to the modulation of the expression of EMT-related genes (E-cadherin, N-cadherin, vimentin, β-catenin, Snail, MMP-2 and MMP-9) in several *in vitro* cancer models.[48] For example, capsaicin is able to decrease the metastatic activity of thyroid cancer cells by altering the expression of EMT markers, such as E-cadherin, MMP-2 and MMP-9.[45] All these observations are closely related to our results.

4. Conclusion

Evidence indicates that MH exerts cytotoxic effects in HCT-116 and LoVo cells inducing oxidative stress which generates ROS, suppressing antioxidant enzyme activity and expression, and promoting DNA, protein and lipid damage. MH treatment suppressed the expression of NF-κB and its related gene p-IκBα in both cell lines, which is an important marker for initiating an inflammatory response in colon cancer. MH acts as a regulator of energy metabolism of HCT-116 and LoVo cells both in anaerobic and aerobic pathways, and indicates an alternative strategy for the treatment of cancer. Interestingly, MH can suppress the AMPK pathway, which plays an important role in mitochondrial biogenesis in cancer cells to promote metabolism under glucose limiting conditions. Moreover, MH suppresses the migration and invasion ability in cells tested *in vitro* by reducing the expression of MMP-2 and MMP-9 in a dose-dependent way. Additionally, the expression of EMT-related markers such as E-cadherin, N-cadherin and β-catenin levels is also significantly regulated after MH treatment. This study represents some new findings concerning the efficacy of MH in producing potential anti-cancer and anti-metastasis effects in colon cancer cells. Therefore, future research is needed to authenticate the chemopreventive approach in *in vivo* models.

Abbreviations

AMPK	AMP-activated protein kinases
CRC	Colorectal cancer
2-DG	2-Deoxy-D-glucose
2,4-DNP	2,4-Dinitrophenol
ECAR	Extracellular acidification rate
EMT	Epithelial–mesenchymal transition
GPx	Glutathione peroxidase
GR	Glutathione reductase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HO-1	Heme oxygenase 1
IκB	Inhibitor of kappa B
MH	Manuka honey
MMP	Matrix metalloproteinases
mpH	Milli-pH units
MRC	Maximum respiration rate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
Nrf2	Nuclear factor E2-related factor 2
OCR	Oxygen consumption rate
OGG1	8-Oxoguanine DNA glycosylase
OXPHOS	Oxidative phosphorylation
PGC1α	Peroxisome proliferator-activated receptor c coactivator 1 alpha
Pmole/min	Picomoles per minute
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SRC	Spare respiratory capacity
TBARS	Thiobarbituric acid-reactive substance
TBST	Tris HCI buffered saline with Tween 20

Conflicts of interest

The authors declare no conflicts of interest.

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References

1 R. L. Siegel, K. D. Miller, S. A. Fedewa, D. J. Ahnen, R. G. S. Meester, A. Barzi and A. Jemal, Colorectal cancer statistics, 2017, *CA-Cancer J. Clin.*, 2017, **67**, 177–193.
2 J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman and F. Bray, Cancer incidence and mortality worldwide: sources, methods and

major patterns in GLOBOCAN 2012, *Int. J. Cancer*, 2015, **136**, E359–E386.
3 V. Nogueira and N. Hay, Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy, *Clin. Cancer Res.*, 2013, **19**, 4309–4314.
4 S. K. Jaganathan and M. Mandal, Involvement of non-protein thiols, mitochondrial dysfunction, reactive oxygen species and p53 in honey-induced apoptosis, *Invest. New Drugs*, 2010, **28**, 624–633.
5 S. Gunasekaran, K. Venkatachalam and N. Namasivayam, p-Methoxycinnamic acid, an active phenylpropanoid induces mitochondrial mediated apoptosis in HCT-116 human colon adenocarcinoma cell line, *Environ. Toxicol. Pharmacol.*, 2015, **40**, 966–974.
6 J. C. Liao, K. T. Lee, B. J. You, C. L. Lee, W. T. Chang, Y. C. Wu and H.-Z. Lee, Raf/ERK/Nrf2 signaling pathway and MMP-7 expression involvement in the trigonelline-mediated inhibition of hepatocarcinoma cell migration, *Food Nutr. Res.*, 2015, **59**, 29884.
7 M. Bhardwaj, N.-H. Kim, S. Paul, R. Jakhar, J. Han and S. C. Kang, 5-Hydroxy-7-methoxyflavone triggers mitochondrial-associated cell death via reactive oxygen species signaling in human colon carcinoma cells, *PLoS One*, 2016, **11**, e0154525.
8 S. Yang, H. Zhang, X. Yang, Y. Zhu and M. Zhang, Evaluation of antioxidative and antitumor activities of extracted flavonoids from Pink Lady apples in human colon and breast cancer cell lines, *Food Funct.*, 2015, **6**, 3789–3798.
9 L. Sun, C. Luo and J. Liu, Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation, *Food Funct.*, 2014, **5**, 1909–1914.
10 S. B. Raja, V. Rajendiran, N. K. Kasinathan, P. Amrithalakshmi, S. Venkatabalasubramanian, M. R. Murali, H. Devaraj and S. N. Devaraj, Differential cytotoxic activity of Quercetin on colonic cancer cells depends on ROS generation through COX-2 expression, *Food Chem. Toxicol.*, 2017, **106**, 92–106.
11 A. R. Diers, K. A. Broniowska, C.-F. Chang and N. Hogg, Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition, *Biochem. J.*, 2012, **444**, 561–571.
12 B. Chaube, P. Malvi, S. V. Singh, N. Mohammad, B. Viollet and M. K. Bhat, AMPK maintains energy homeostasis and survival in cancer cells via regulating p38/PGC-1α-mediated mitochondrial biogenesis, *Cell Death Dis.*, 2015, **1**, 15063.
13 S.-M. Jeon, Regulation and function of AMPK in physiology and diseases, *Exp. Mol. Med.*, 2016, **48**, e245.
14 B. Boyer and J. P. Thiery, Epithelium-mesenchyme interconversion as example of epithelial plasticity, *APMIS*, 1993, **101**, 257–268.
15 G. P. Gupta and J. Massagué, Cancer metastasis: building a framework, *Cell*, 2006, **127**, 679–695.
16 O. R. F. Mook, W. M. Frederiks and C. J. F. Van Noorden, The role of gelatinases in colorectal cancer progression and metastasis, *Biochim. Biophys. Acta*, 2004, **1705**, 69–89.
17 L. Song, J. Chang and Z. Li, A serine protease extracted from *Trichosanthes kirilowi*i, induces apoptosis via the PI3 K/AKT-mediated mitochondrial pathway in human colorectal adenocarcinoma cells, *Food Funct.*, 2016, **7**, 843–854.
18 J. M. Alvarez-Suarez, M. Gasparrini, T. Y. Forbes-Hernández, L. Mazzoni and F. Giampieri, The composition and biological activity of honey: a focus on Manuka honey, *Foods*, 2014, **3**, 420–432.
19 J. M. Alvarez-Suarez, F. Giampieri, M. Cordero, M. Gasparrini, T. Y. Forbes-Hernández, L. Mazzoni, S. Afrin, P. Beltrán-Ayala, A. M. González-Paramás and C. Santos-Buelga, Activation of AMPK/Nrf2 signalling by Manuka honey protects human dermal fibroblasts against oxidative damage by improving antioxidant response and mitochondrial function promoting wound healing, *J. Funct. Foods*, 2016, **25**, 38–49.
20 S. Afrin, T. Y. Forbes-Hernandez, M. Gasparrini, S. Bompadre, J. L. Quiles, G. Sanna, N. Spano, F. Giampieri and M. Battino, Strawberry-Tree Honey Induces Growth Inhibition of Human Colon Cancer Cells and Increases ROS Generation: A Comparison with Manuka Honey, *Int. J. Mol. Sci.*, 2017, **18**, 613.
21 M. J. Fernandez-Cabezudo, R. El-Kharrag, F. Torab, G. Bashir, J. A. George, H. El-Taji and B. K. Al-Ramadi, Intravenous administration of manuka honey inhibits tumor growth and improves host survival when used in combination with chemotherapy in a melanoma mouse model, *PLoS One*, 2013, **8**, e55993.
22 F. Giampieri, J. L. Quiles, F. J. Orantes-Bermejo, M. Gasparrini, T. Y. Forbes-Hernandez, C. Sánchez-González, J. Llopis, L. Rivas-García, S. Afrin, A. Varela-López, D. Cianciosi, P. Reboredo-Rodriguez, C. T. Fernández-Piñar, C. Iglesias Ruben, R. Ruiz, S. Aparicio, J. Crespo, D. L. Lopez, J. Xiao and M. Battino, Are by-products from beeswax recycling process a new promising source of bioactive compounds with biomedical properties?, *Food Chem. Toxicol.*, 2018, **112**, 126–133.
23 F. Giampieri, J. M. Alvarez-Suarez, M. Gasparrini, T. Y. Forbes-Hernandez, S. Afrin, S. Bompadre, C. Rubini, A. Zizzi, P. Astolfi and C. Santos-Buelga, Strawberry consumption alleviates doxorubicin-induced toxicity by suppressing oxidative stress, *Food Chem. Toxicol.*, 2016, **94**, 128–137.
24 Y. Shen, J. Yang, J. Li, X. Shi, L. Ouyang, Y. Tian and J. Lu, Carnosine inhibits the proliferation of human gastric cancer SGC-7901 cells through both of the mitochondrial respiration and glycolysis pathways, *PLoS One*, 2014, **9**, e104632.
25 B. N. Waghela, A. Sharma, S. Dhumale, S. M. Pandey and C. Pathak, Curcumin conjugated with PLGA potentiates sustainability, anti-proliferative activity and apoptosis in human colon carcinoma cells, *PLoS One*, 2015, **10**, e0117526.
26 S. Amatori, L. Mazzoni, J. M. Alvarez-Suarez, F. Giampieri, M. Gasparrini, T. Y. Forbes-Hernandez, S. Afrin,

A. E. Provenzano, G. Persico and B. Mezzetti, Polyphenol-rich strawberry extract (PRSE) shows in vitro and in vivo biological activity against invasive breast cancer cells, *Sci. Rep.*, 2016, **6**, 30917.

27 M. L. Circu and T. Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, *Free Radicals Biol. Med.*, 2010, **48**, 749–762.

28 S. Shan, J. Shi, Z. Li, H. Gao, T. Shi, Z. Li and Z. Li, Targeted anti-colon cancer activities of a millet bran-derived peroxidase were mediated by elevated ROS generation, *Food Funct.*, 2015, **6**, 2331–2338.

29 I. Khan, S. Paul, R. Jakhar, M. Bhardwaj, J. Han and S. C. Kang, Novel quercetin derivative TEF induces ER stress and mitochondria-mediated apoptosis in human colon cancer HCT-116 cells, *Biomed. Pharmacother.*, 2016, **84**, 789–799.

30 S. B. Raja, V. Rajendiran, N. K. Kasinathan, P. Amrithalakshmi, S. Venkatabalasubramanian, M. R. Murali, H. Devaraj and S. N. Devaraj, Differential cytotoxic activity of Quercetin on colonic cancer cells depends on ROS generation through COX-2 expression, *Food Chem. Toxicol.*, 2017, **106**, 92–106.

31 A. L. Jacobs and P. Schär, DNA glycosylases: in DNA repair and beyond, *Chromosoma*, 2012, **121**, 1–20.

32 E. F. R. Nascimento, M. L. Ribeiro, D. O. Magro, J. Carvalho, D. T. Kanno, C. A. R. Martinez and C. S. R. Coy, Tissue expression of the genes MUTYH and OGG1 in patients with sporadic colorectal cancer, *Arq. Bras. Cir. Dig.*, 2017, **30**, 98–102.

33 M. J. Morgan and Z.-g. Liu, Crosstalk of reactive oxygen species and NF-Î°B signaling, *Cell Res.*, 2015, **21**, 103–115.

34 K. Sakamoto, S. Maeda, Y. Hikiba, H. Nakagawa, Y. Hayakawa, W. Shibata, A. Yanai, K. Ogura and M. Omata, Constitutive NF-κB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth, *Clin. Cancer Res.*, 2009, **15**, 2248–2258.

35 V. P. Venancio, P. A. Cipriano, H. Kim, L. M. G. Antunes, S. T. Talcott and S. U. Mertens-Talcott, Cocoplum (*Chrysobalanus icaco*, L.) anthocyanins exert anti-inflammatory activity in human colon cancer and non-malignant colon cells, *Food Funct.*, 2017, **8**, 307–314.

36 M. Kobayashi and M. Yamamoto, Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation, *Antioxid. Redox Signaling*, 2005, **7**, 385–394.

37 S. Menegon, A. Columbano and S. Giordano, The dual roles of NRF2 in cancer, *Trends Mol. Med.*, 2016, **22**, 578–593.

38 M. Wu, A. Neilson, A. L. Swift, R. Moran, J. Tamagnine, D. Parslow, S. Armistead, K. Lemire, J. Orrell and J. Teich, Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells, *Am. J. Physiol.: Cell Physiol.*, 2007, **292**, C125–C136.

39 M. S. Islam, F. Giampieri, M. Janjusevic, M. Gasparrini, T. Y. Forbes-Hernandez, L. Mazzoni, S. Greco, S. R. Giannubilo, A. Ciavattini, B. Mezzetti, F. Capocasa, M. Castellucci, M. Battino and P. Ciarmela, An anthocyanin rich strawberry extract induces apoptosis and ROS while decreases glycolysis and fibrosis in human uterine leiomyoma cells, *Oncotarget*, 2017, **8**, 23575.

40 H.-S. Kim, T. Wannatung, S. Lee, W. K. Yang, S. H. Chung, J.-S. Lim, W. Choe, I. Kang, S.-S. Kim and J. Ha, Quercetin enhances hypoxia-mediated apoptosis via direct inhibition of AMPK activity in HCT116 colon cancer, *Apoptosis*, 2012, **17**, 938–949.

41 A. Chalkiadaki and L. Guarente, The multifaceted functions of sirtuins in cancer, *Nat. Rev. Cancer*, 2015, **15**, 608–624.

42 X. Chen, K. Sun, S. Jiao, N. Cai, X. Zhao, H. Zou, Y. Xie, Z. Wang, M. Zhong and L. Wei, High levels of SIRT1 expression enhance tumorigenesis and associate with a poor prognosis of colorectal carcinoma patients, *Sci. Rep.*, 2014, **4**, 7481.

43 W. Nutakul, H. S. Sobers, P. Qiu, P. Dong, E. A. Decker, D. J. McClements and H. Xiao, Inhibitory effects of resveratrol and pterostilbene on human colon cancer cells: a side-by-side comparison, *J. Agric. Food Chem.*, 2011, **59**, 10964–10970.

44 J.-Y. Kee, Y.-H. Han, D.-S. Kim, J.-G. Mun, J. Park, M.-Y. Jeong, J.-Y. Um and S.-H. Hong, Inhibitory effect of quercetin on colorectal lung metastasis through inducing apoptosis, and suppression of metastatic ability, *Phytomedicine*, 2016, **23**, 1680–1690.

45 S. Xu, L. Zhang, X. Cheng, H. Yu, J. Bao and R. Lu, Capsaicin inhibits the metastasis of human papillary thyroid carcinoma BCPAP cells through the modulation of the TRPV1 channel, *Food Funct.*, 2018, **9**, 344–354.

46 C.-Y. Zhang, L. Zhang, H.-X. Yu, J.-D. Bao, Z. Sun and R.-R. Lu, Curcumin inhibits invasion and metastasis in K1 papillary thyroid cancer cells, *Food Chem.*, 2013, **139**, 1021–1028.

47 J. Moskwa, M. H. Borawska, R. Markiewicz-Zukowska, A. Puscion-Jakubik, S. K. Naliwajko, K. Socha and J. Soroczynska, Polish natural bee honeys are anti-proliferative and anti-metastatic agents in human glioblastoma multiforme U87MG cell line, *PLoS One*, 2014, **9**, e90533.

48 E.-K. Kim, E.-J. Choi and T. Debnath, Role of phytochemicals in the inhibition of epithelial–mesenchymal transition in cancer metastasis, *Food Funct.*, 2016, **7**, 3677–3685.